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Effect of Exopolysaccharides on Phage-Host Interactions in *Lactococcus lactis*

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In this study, we report that *Lactococcus lactis* strains producing exopolysaccharides (EPS) are sensitive to virulent phages. Eight distinct lytic phages (Q61 to Q68) specifically infecting Eps$^+$ strains were isolated in 47 buttermilk samples obtained from 13 North American factories. The eight phages were classified within the 936 species by the multiplex PCR method, indicating that these phages are not fundamentally distinct from those infecting Eps$^-$ *L. lactis* strains. The host range of these phages was determined with 19 *Lactococcus* strains, including 7 Eps$^+$ and 12 Eps$^-$ cultures. Three phages (Q62, Q63, and Q64) attacked only the Eps$^+$ strain SMQ-419, whereas the five other phages (Q61, Q65, Q66, Q67, and Q68) infected only the Eps$^+$ strain SMQ-420. The five other Eps$^+$ strains (H414, MLT2, MLT3, SMQ-461, and SMQ-575) as well as the 12 Eps$^-$ strains were insensitive to these phages. The monosaccharide composition of the polymer produced by the seven Eps$^+$ strains was determined. The EPS produced by strains MLT3, SMQ-419, and SMQ-575 contained glucose, galactose, and rhamnose. The EPS fabricated by H414 contained only galactose. The EPS made by MLT2, SMQ-420, and SMQ-461 contained glucose and galactose. These findings indicate that the sugar composition of the EPS has no effect on phage sensitivity. The plasmid encoding the *eps* operon was cured from the two phage-sensitive strains. The cured derivatives were still phage sensitive, which indicates that EPS are not necessary for phage infection. Phage adsorption assays showed that the production of EPS does not confer a significant phage resistance phenotype.

Lactococcus lactis is a gram-positive lactic acid bacterium (LAB) used to acidify milk during the manufacture of fermented dairy products. Most *Lactococcus* strains contain numerous plasmids that encode key industrial phenotypes, including the production of extracellular polysaccharides (EPS). When produced in situ, these loosely bound exopolysaccharides decrease the syneresis, increase the viscosity, and improve the texture of dairy products. From a microbiological standpoint, EPS may also protect the producing cells against detrimental environmental conditions. The lactococcal EPS are heteropolysaccharides composed of repeating units of sugars in which galactose, glucose, and rhamnose are the most common carbohydrates. The structure and composition of several lactococcal EPS, as well as the sequence and organization of the *eps* genes, have also been resolved during the last decade (10, 13, 24, 34, 35, 36).

Lytic phages are the most significant cause of fermentation failures in the dairy industry worldwide (21). Lactococcal phages are currently classified into 12 distinct species (16), although a reduction to 11 species was recently proposed (18). However, only three species are predominant in most dairy plants: 936, c2, and P335 (3, 16–18, 22, 23). These three species are members of the *Siphoviridae* family and share some important features, including a double-stranded DNA genome and a long noncontractile tail (1, 16). The EPS-producing *Lactococcus* strains are increasingly used in the dairy industry, and it is well documented that extensive use of a particular strain will lead to milk fermentation failures due to virulent bacteriophages. Yet, to our knowledge, there are only two studies in the literature that mention lactococcal phages infecting EPS-producing strains (28, 31). In fact, a total of only three phages were described in these two studies conducted in the 1970s. The first study described the isolation of phages sl60 and sl122, but unfortunately, they were not characterized further (31). The second study described the isolation of phage KSY1, which belongs to the *Podoviridae* family, from the Finnish fermented milk called "viili" (28). KSY1 is the reference phage of the lactococcal phage species that bears its name. This morphotype is very rare, because only two KSY1-like phages have been reported in lactococci (28, 29). These results suggested that the lactococcal phages infecting EPS-producing strains may be different from those infecting non-EPS-producing strains, which are traditionally used in the manufacture of most fermented dairy products.

In the mid-1980s, Saxelin et al. (29) isolated and characterized by electron microscopy and host range 13 morphological types of phages from viili. However, it is not known whether these phages infected EPS-producing strains (29). Moineau et al. (22) showed that none of the 27 distinct lactococcal phages isolated from North American buttermilk factories could propagate on six EPS-producing strains. These results suggested that EPS production could protect cells against lytic phage infection (22). In fact, a number of authors have suggested that EPS may protect LAB against phages (7, 10, 12, 19, 22, 37). Looijesteijn et al. (19) demonstrated that cell-associated EPS

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TABLE 1. Bacterial strains and bacteriophages used in this study

Strains or phages	Revelant characteristics[a]	Source or reference
Strains		
L. lactis		
SMQ-5	LM0230, plasmid free, Str^r Fus^r Lac$^-$ Eps$^-$	20; this study
SMQ-419	Lac$^+$ Eps$^+$, industrial strain	This study
SMQ-420	Lac$^+$ Eps$^+$, industrial strain	This study
SMQ-461	Lac$^+$ Eps$^+$, raw milk isolate	This study
SMQ-575	Lac$^+$ Eps$^+$, industrial strain	This study
H414	Lac$^+$ Eps$^+$, industrial strain	13, 36
MLT2	Lac$^+$ Eps$^+$, industrial strain	36
MLT3	Lac$^+$ Eps$^+$, industrial strain	36
IL1403	Laboratory strain, plasmid free	4
SMQ-737	SMQ-419 cured of EPS production, Lac$^+$ Eps$^-$	This study
SMQ-738	SMQ-420 cured of EPS production, Lac$^+$ Eps$^-$	This study
S. thermophilus SMQ-301	Industrial strain, Lac$^+$, host for phage DT1	33
Phages		
Q61	Small isometric head, 936 species, U.S.	This study
Q62	Small isometric head, 936 species, U.S.	This study
Q63	Small isometric head, 936 species, Canada	This study
Q64	Small isometric head, 936 species, U.S.	This study
Q65	Small isometric head, 936 species, U.S.	This study
Q66	Small isometric head, 936 species, U.S.	This study
Q67	Small isometric head, 936 species, U.S., Canada	This study
Q68	Small isometric head, 936 species, U.S.	This study
P008	Small isometric head, 936 species, Germany	H.-W. Ackermann[b]
c2	Prolate head, c2 species, U.S.	20
P335	Small isometric head, P335 species, Germany	5

[a] Lac$^+$, lactose-fermenting ability; Eps$^+$, EPS-producing phenotype; Str^r, streptomycin resistance; Fus^r, fusidic acid resistance.
[b] Université Laval.

granted lactococcal strains a slight protection against phages. Forde and Fitzgerald (12) revealed that loosely bound EPS produced by some *Lactococcus* strains partially inhibited phage adsorption, possibly by masking phage receptors. However, today, some EPS-producing lactococcal strains appear to be infected by phages in commercial applications such as buttermilk. Thus, the involvement of EPS in the phage infection process of LAB is still unclear.

In gram-negative bacteria, EPS and/or capsular polysaccharide may be directly involved in phage-host interactions. Specific phages infecting *Escherichia coli* and *Vibrio cholerae* strains that possess polysaccharide capsule have been isolated (2, 8, 30). In *Rhizobium meliloti*, the EPS production has been shown to inhibit phage adsorption (9).

The aim of this study was to characterize bacteriophages specifically infecting EPS-producing *L. lactis* strains and to study the effect of EPS on phage-host interactions.

MATERIALS AND METHODS

Bacterial strains and media. The *L. lactis* strains used in this study are listed in Table 1 and were grown at 30°C in M17 broth (32) supplemented with 0.5% glucose (GM17) or lactose (LM17) (Quélab, Montréal, Québec, Canada). The species and subspecies of lactococcal strains were confirmed as outlined previously (3, 26) with the reference strains *L. lactis* subsp. *cremoris* SMQ-5, *L. lactis* subsp. *lactis* IL-1403, and *Streptococcus thermophilus* SMQ-301. The seven Eps$^+$ strains were genotypically identified as *L. lactis* subsp. *cremoris*.

Phage isolation and classification. Forty-seven phage-containing samples from 13 North American buttermilk factories were provided by Quest International. The presence of phages in these samples was confirmed by spot test on seven EPS-producing *Lactococcus* strains as described elsewhere (22, 23). For each sample, five well-defined plaques were isolated, and high phage titers were obtained by the method of Jarvis (15). This procedure was repeated twice. The newly isolated phages were designated with the prefix "Q." All phages used in this study are listed in Table 1. These newly isolated lactococcal phages were classified within a phage species by the multiplex PCR method (17). Phage P008 was used as the reference for the 936 species, phage c2 was used as the reference for the c2 species, and phage P335 was used as the reference for the P335 species. Phage morphology was observed with a Phillips 420 transmission electron microscope at 80 kV as described previously (23). The host range of the isolated phages was determined on 19 industrial *L. lactis* strains, which included 7 EPS-producing strains (Eps$^+$) and 12 EPS-negative strains (Eps$^-$), as described previously (23).

Phage DNA isolation. The Maxi Lambda DNA purification kit (Qiagen, Chatsworth, Calif.) was used for lactococcal phage DNA purification with the following modifications: L2 buffer was adjusted to provide a final concentration of 10% polyethylene glycol and 0.5 M sodium chloride. Proteinase K (100 μl of a 20-mg/ml solution) was added before the L4 buffer. The mixture was incubated for 30 min at 55°C. DNA was resuspended in 100 μl of 10 mM Tris-HCl (pH 7.8) and stored at −20°C for later use.

DNA manipulations. Plasmid DNA was purified by the modified method of O'Sullivan and Klaenhammer (25). Total DNA of *L. lactis* strains was extracted by the method described by Hill et al. (14) with some modifications. The Tris-EDTA (TE)-saturated phenol was replaced by two steps involving an equal volume of phenol-chloroform. One-fifth volume of 7.5 M ammonium acetate was added, and the DNA was precipitated by the addition of 2 volumes of cold 95% ethanol. Restriction endonucleases (Roche Diagnostics, Laval, Québec, Canada) were used as recommended by the manufacturer. After restriction, phage DNA samples were heated for 10 min at 70°C to avoid possible cohesive end ligation. DNA was electrophoresed on 0.8% agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and visualized by UV photography after staining in ethidium bromide.

Analysis of EPS composition. For each strain, 1 liter of LM17 was inoculated with an overnight culture (1% [vol/vol]) and incubated at 25°C for 48 h. The EPS was extracted by the method of Cerning (6) and further purified on a Sephacryl S-400 HR column by elution with 50 mM NH_4HCO_3. The polymer (1 mg) was hydrolyzed (2 M trifluoroacetic acid, 1 h, 121°C) and analyzed by high-perfor-

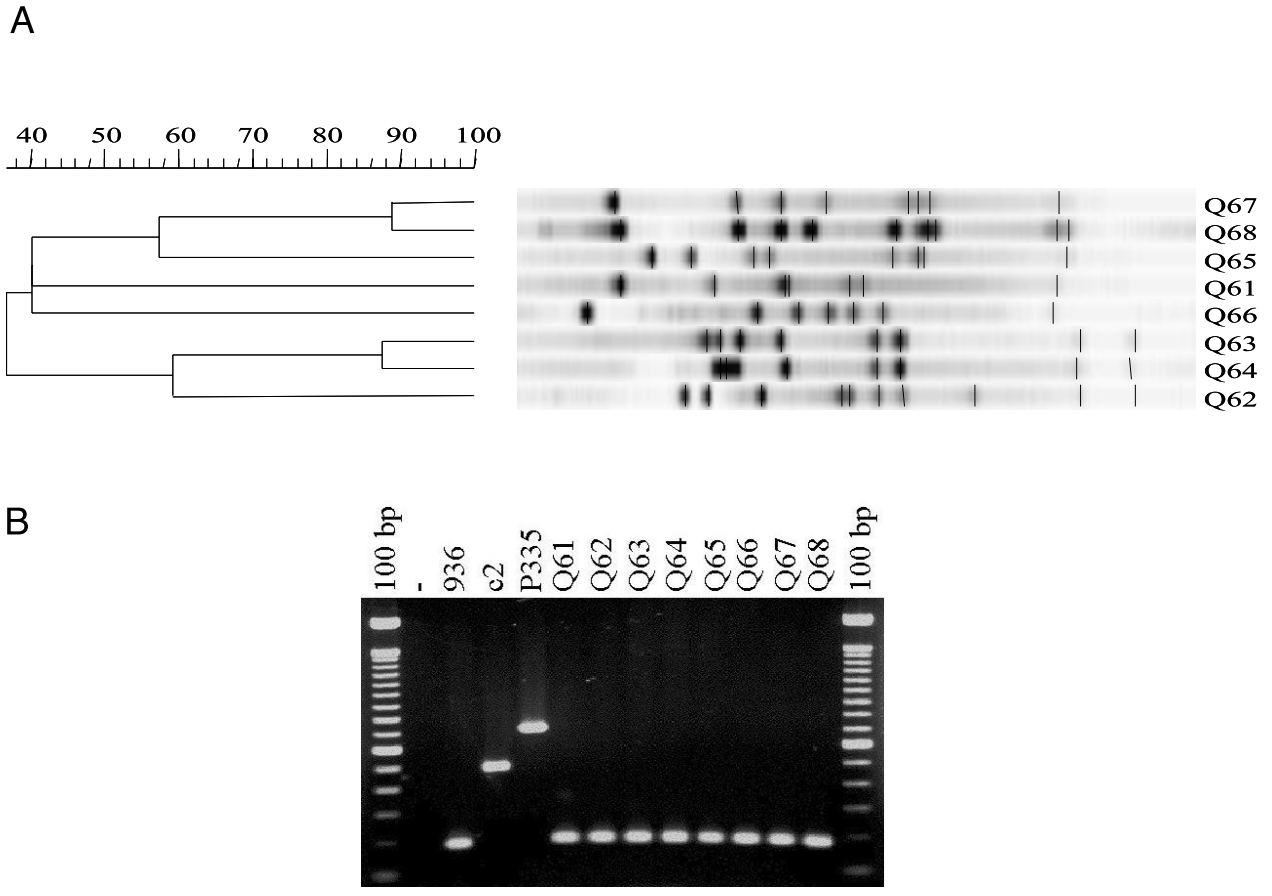

FIG. 1. Characterization of lactococcal phages Q61 to Q68. (A) Dendrogram of *Eco*RV restriction profiles of the eight lactococcal phages. (B) Classification of the eight phages by multiplex PCR.

mance liquid chromatography (HPLC) at 65°C on a 30-cm by 7.8-mm Aminex-87H column (Bio-Rad, Mississauga, Ontario, Canada), eluted at 0.6 ml/min with 0.005 N sulfuric acid. The sugars were detected by refractive index monitoring at 35°C.

Plasmid curing and microbiological assays. The *L. lactis* Eps^+ strains SMQ-419 and SMQ-420 were cured of the plasmid containing the *eps* operon by serial transfers in GM17 broth. After 1 week, the culture was streaked, and isolated colonies were transferred in milk and incubated for 16 h at 25°C. Strains were regarded as ropy if strings of 5 mm or more were detected when the culture was touched with a sterile pipette. The plasmid and total DNA of nonmucoid isolates were extracted, and the absence or presence of the *eps* gene cluster was verified by Southern analysis. Efficiency of plating was performed on Eps^- and Eps^+ isolates as described previously (27). Phage adsorption tests were carried out on the *L. lactis* strains SMQ-419, SMQ-420, SMQ-737, and SMQ-738, as described previously (11), but with the following modifications: GM17 broth was used, and the incubation temperature was 30°C. Phage adsorption assays were also performed on bacterial cells washed twice with 0.1% peptone (9).

Detection of the *eps* gene cluster. Plasmid and chromosomal DNAs were transferred onto positively charged nylon membranes by capillary blotting. The probe was constructed by labeling PCR fragments with the Dig High-Prime labeling kit (Roche). The probe was made of a PCR product corresponding to the *epsD* (priming glycosyltransferase) gene (primers 5′-TGATCCCCGTGTAA CGAAGA-3′ and 5′-AAGAGAGGCGCTCCCCATAT-3′). Prehybridization, hybridization, washes, and detection by chemiluminescence were performed as suggested by the manufacturer (Roche).

RESULTS AND DISCUSSION

Phage isolation. The phage content of 47 buttermilk samples from 13 dairy manufacturing plants was examined by analyzing a total of 235 single plaques (5 plaques per sample) propagated on EPS-producing *L. lactis* strains. Based on DNA restriction profiles with *Eco*RV (Fig. 1) and *Eco*RI (data not shown), only eight different phages were found among the 235 isolates. Furthermore, some of these phages were very similar. Only one phage was isolated from the samples provided by four factories. In the nine remaining factories, between two and four different lactococcal phages were found. A buttermilk manufacturing facility can therefore harbor many distinct phages. Phages with identical DNA restriction profiles were also detected in many factories. For example, phage Q67 was isolated in six factories and Q64 was found in 8 of 13 dairies, indicating that a specific phage can be found in several geographic locations. Three phages (Q63, Q66, and Q68) were unique to one plant. The isolation of multiple lactococcal phages within one plant was not surprising, because a similar phenomenon was previously observed in cheese plants (3). Because these facilities used similar and application-oriented starter cultures and the use of specific strains will partly dictate the presence of particular phages within a dairy plant, these eight phages appear fit to propagate under the buttermilk manufacturing conditions. These results also unequivocally showed that EPS-producing lactococcal strains can be infected by phages during buttermilk production.

TABLE 2. Classification of EPS produced by seven strains and phage sensitivity of these strains

Group	Strains	Phages	Molar ratio of sugar[a]		
			Gal	Glc	Rha
I	MLT3		1	1.8	0.9
	SMQ-575		1	2.0	1.1
	SMQ-419	Q62, Q63, Q64	1	1.4	0.6
II	H414		1	0.1	0.0
III	MLT2		1	1.8	0.0
	SMQ-420	Q61, Q65, Q66, Q67, Q68	1	1.6	0.0
IV	SMQ-461		1	2.0	0.0

[a] Gal, galactose; Glc, glucose; Rha, rhamnose.

TABLE 3. EOP of phages Q61 to Q68 on *L. lactis* SMQ-737 and SMQ-738

Phage	EOP[a]:	
	SMQ-737	SMQ-738
Q61		1.20 ± 0.09
Q62	0.71 ± 0.21	
Q63	0.69 ± 0.06	
Q64	0.96 ± 0.24	
Q65		1.05 ± 0.15
Q66		1.03 ± 0.14
Q67		1.05 ± 0.25
Q68		1.10 ± 0.12

[a] The EOP of phages Q62, Q63, and Q64 is 1.0 on SMQ-419, and the EOP of phages Q61, Q65, Q66, Q67, and Q68 is 1.0 on *L. lactis* SMQ-420 ($n = 3$).

Phage classification. The multiplex PCR method was performed to catalogue the eight newly isolated phages within the three most common lactococcal phage species found in dairy plants, namely, 936, c2, and P335 (17, 21). The eight phages isolated in this study gave a PCR product slightly lower than 200 bp, indicating that they all belong to the species 936 (Fig. 1). The typical 936 morphology (isometric head and noncontractile tail) was confirmed by electron microscopy (data not shown). These results indicate that these phages are not fundamentally distinct from those infecting Eps$^-$ *L. lactis* strains. They also suggest that the previous isolation of phages from the KSY1 species may indeed be a very rare event (28, 29).

Host range. The host range of the eight phages was determined on 19 lactococcal strains, which included 7 Eps$^+$ strains and 12 Eps$^-$ strains. These strains are currently used in various combinations within several commercial starter cultures (3 strains per starter) for buttermilk production. All 12 Eps$^-$ strains were insensitive to the eight phages. Only two of seven mucoid strains were sensitive to these phages (Table 2). *L. lactis* SMQ-419 was sensitive to phages Q62, Q63, and Q64, whereas *L. lactis* SMQ-420 was sensitive to phages Q61, Q65, Q66, Q67, and Q68. These results show that the eight phages are specific to two Eps$^+$ *L. lactis* strains.

Analysis of the EPS composition. The EPS produced by the seven EPS-producing strains was purified, hydrolyzed, and analyzed by HPLC for their monosaccharide composition. The *L. lactis* Eps$^+$ strains MLT3 (group I), H414 (group II), and MLT2 (group III) were used as reference strains in accordance with the classification of van Kranenburg et al. (36). The monosaccharide composition of the EPS produced by the three reference strains was in agreement with those reported in the literature (36). The EPS produced by strains MLT3, SMQ-419, and SMQ-575 (group I) contained glucose, galactose, and rhamnose (Table 2). The EPS fabricated by strain H414 (group II) contained only galactose. The EPS made by strains MLT2 and SMQ-420 (group III) contained glucose and galactose. The EPS assembled by strain SMQ-461 also contained only glucose and galactose, but preliminary nuclear magnetic resonance analysis showed that the structure of this EPS is different from the one produced by group III, and it may belong to another group (data not shown). Given that phage-sensitive and phage-insensitive strains were found to produce EPS that belong to either group I (glucose, galactose, and rhamnose) or group III (glucose and galactose), these results clearly indicate that the monosaccharide composition of the EPS has no influence on the phage sensitivity of an *L. lactis* strain.

Effect of EPS on phage sensitivity. The phage-sensitive strains SMQ-419 and SMQ-420 were cured of the plasmid coding for the EPS production, and the cured derivatives were named SMQ-737 and SMQ-738, respectively. The absence of these plasmids in the cured derivatives was verified by plasmid profile analysis, and the absence of the *eps* operon was confirmed by Southern analysis in which an *epsD* (priming glycosyltransferase) probe failed to hybridize with total DNA (data not shown). Both cured derivatives exhibited the same phage sensitivity as that of their parents, indicating that the EPS is not necessary for phage infection. The efficiency of plating (EOP; phage titer on the Eps$^+$ strain divided by the phage titer on the Eps$^-$ strain) of these phages was also measured (Table 3). The EOP of phages Q61, Q65, Q66, Q67 and Q68 were 1.0, indicating the plasmid encoding the *eps* operon in *L. lactis* SMQ-420 does not confer a phage resistance phenotype. By association, these data also indicated that the EPS produced by SMQ-420 (containing glucose and galactose) does not prevent phage infection. The EOP of phages Q62 and Q63 were slightly below 1.0, indicating that the plasmid encoding the *eps* operon in *L. lactis* SMQ-419 may arguably provide weak resistance against these two phages. This limited antiviral phenotype is likely due to the production of the EPS (containing glucose, galactose, and rhamnose).

Effect of EPS on phage adsorption. The percentages of phage adsorption on wild-type strains (Eps$^+$) and cured derivatives (Eps$^-$) were compared. No significant differences were observed between the phage adsorption on SMQ-420 (Eps$^+$) and that on SMQ-738 (Eps$^-$) (data not shown). The adsorption of phages Q62, Q63, and Q64 was somewhat better on the SMQ-737 strain (Eps$^-$) than on the parent Eps$^+$ strain SMQ-419 (Table 4). The standard deviations were relatively high (13 to 15%) for the phage adsorption results with the Eps$^+$ strain *L. lactis* SMQ-419 compared to similar data from the Eps$^-$ strain SMQ-737 (0.6 to 4%). When the cells were washed to remove the loosely bound EPS, the percentages of phage adsorption were similar on both strains, and the standard deviations were lower (between 3.6 and 5.0%) (Table 4). The variability in the phage adsorption assays may be explained by the fluctuating EPS production. These results indicate that the

TABLE 4. Adsorption percentage of phages Q62, Q63, and Q64 on *L. lactis* SMQ-419 and SMQ-737

Phage	% Adsorption of phage[a] on:			
	Unwashed cells		Washed cells	
	SMQ-419	SMQ-737	SMQ-419	SMQ-737
Q62	70.3 ± 14.7	89.5 ± 0.8	95.2 ± 3.6	95.0 ± 5.5
Q63	86.0 ± 13.2	95.5 ± 4.0	95.2 ± 3.9	97.7 ± 1.3
Q64	83.1 ± 15.2	95.3 ± 0.6	95.8 ± 5.0	96.6 ± 1.8

[a] $n = 3$.

EPS is likely responsible for the difference between the adsorption on SMQ-419 and that on SMQ-737. Moreover, these data confirm that the weak resistance against phages Q62 and Q63 is conferred by the EPS. These results are in agreement with those of Looijesteijn et al. (19), which showed that the production of group I EPS (such as those produced by SMQ-419) confers a marginal phage resistance phenotype (EOP above 0.5).

The production of EPS increases the viscosity of the medium, and perhaps this molecular crowding slightly affects the spread of phage infection. It also possible that the EPS could protect the strain by coating the cell, but this protection would be limited because the lactococcal EPS is loosely bound. Nonetheless, it is clear from the evidence presented above that processors should not rely on the production of EPS to protect starter cultures against phages. It remains to be seen if the structure of some lactococcal EPS could be involved in phage sensitivity or insensitivity. Finally, the mine if other phage resistance mechanisms are present in the phage-insensitive Eps^+ strains. Finally, the phage insensitivity reported here may simply be related to the absence of specific phage receptors on the cell surface of these strains. Further research is under way to address these issues. To our knowledge, this is the first thorough analysis of phages infecting EPS-producing gram-positive bacteria.

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